

RENAL FUNCTION IN MARINE TELEOSTS

II. THE NITROGENOUS CONSTITUENTS OF THE URINE OF SCULPIN AND FLOUNDER, WITH PARTICULAR REFERENCE TO TRIMETHYLAMINE OXIDE

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In view of the rather extensive work which has been done upon kidney function in the sculpin (*Myoxocephalus octodecimspinosus*), a common marine teleost (for review of literature see 34), it became of interest to investigate more fully the nitrogenous constituents of the urine. For comparison the flounder (*Pseudopleuronectes americanus*) was likewise studied. Analysis of samples of urine from these two species for the ordinary nitrogenous constituents showed that approximately one-half of the total nitrogen was still unaccounted for. The presence of trimethylamine oxide in other marine fishes (see Table III) made it appear likely that this compound might be present in the urine of our animals. Investigation has demonstrated its occurrence in sculpin urine in significant amounts, but its absence in detectable quantities from the urine of the flounder. Brief supplemental studies demonstrated the occurrence of the oxide in the urine of the daddy sculpin (*Myoxocephalus scorpius*) and confirmed its presence in the urine of the goosefish (*Lophius piscatorius*) (18).

MATERIAL AND METHODS

Freshly caught sculpins and flounders were brought into the laboratory, the urinary papilla was tied off, and the fish were transferred to live cars, usually for 24 hours, sometimes longer. At the end of this time they were killed by a blow on the head, and the urine was removed from the exposed bladder by syringe. Following are additional details concerning the urine samples listed in Tables I and II; unless otherwise noted preservation was by toluol and hydrochloric acid. All samples were kept in a refrigerator when not in use. *Sculpin 1*: about 475 cc., from 217 sculpins; chloroform and toluol. *Sculpin 2*: about 475 cc., from 239 sculpins; 1 per cent sulfuric acid. *Sculpin 3*: 15 cc. from a few specimens. *Flounder 1*: about 475 cc., from 135 flounders; chloroform and toluol. *Flounder 2*: about 475 cc.,

¹ With the assistance of Gordon Spence.

from 117 flounders; 1 per cent sulfuric acid. *Flounder 3*: 15 cc. from a few specimens. *Flounder 4*: same. *Daddy sculpin*: from two specimens. *Goosefish*: from one specimen in good condition. All sculpins and all but one group of fifty flounders (taken in net) were caught with hook and line. The urine samples were collected at the Mt. Desert Island Biological Laboratory, Salsbury Cove, Maine.

Total nitrogen was determined by the methods of Van Slyke (49) (*Sculpin 1* and *2*, *Flounder 1* and *2*) and Pregl (39) (all other specimens); ammonia by the method of Folin and Bell (13); urea by the method of Van Slyke (50), using 0.5 M. veronal rather than phosphate as a buffer because of the high magnesium content of the urine; uric acid by the method of Benedict and Franke (4); amino acids by the method of Folin (12); total creatinine by the method of Folin (11); and chloride by the method of Van Slyke (48) as modified by Smith (41). Trimethylamine oxide was determined by the method of Hoppe-Seyler (24).

RESULTS

The analytical data for the ordinary nitrogenous constituents of the large pooled samples of sculpin and flounder urine are summarized in Table I. The chloride values for these samples, in millimols per

TABLE I

Distribution of nitrogenous constituents of sculpin and flounder urine.

	Sculpin 1		Sculpin 2		Flounder 1		Flounder 2	
	Nitrogen	Total nitrogen	Nitrogen	Total nitrogen	Nitrogen	Total nitrogen	Nitrogen	Total nitrogen
	mgm./100 cc.	per cent	mgm./100 cc.	per cent	mgm./100 cc.	per cent	mgm./100 cc.	per cent
Total N.....	88.0		84.5		43.4		84.0	
Urea N.....	13.5	15.3	11.9	14.1	9.2	21.2	11.3	13.5
Ammonia N.....	1.1	1.3	1.7	2.0	0.8	1.8	2.2	2.6
Uric acid N.....	0.6	0.7	0.6	0.7	0.5	1.2	1.1	1.3
Amino acid N....	3.9	4.4	3.4	4.0	4.2	9.7	6.9	8.2
Total creatinine N	19.7	22.4	21.1	25.0	6.5	15.0	21.9	26.1
Undetermined N.	49.2	55.9	45.8	54.2	22.2	51.1	40.6	48.3

liter, are as follows: *Sculpin 1*, 99; *Sculpin 2*, 105; *Flounder 1*, 184; *Flounder 2*, 138. It has previously been demonstrated (15) that sculpins, when handled under rather ideal conditions, with marked precautions to avoid skin injury, regularly show a higher average urinary total nitrogen and a lower average urinary chloride (see also 16) than those reported here. With repeated handling under the usual experimental conditions, the animals typically develop a diuresis, which is characterized by a rather rapid decrease in urinary con-

centration of total nitrogen and a sharp increase in the urinary concentration of chloride (15, 37). Under the circumstances, the divergence in the present samples from the more ideal values can undoubtedly be attributed to the manner in which the sculpins were handled. With the large number of animals used, the handling was necessarily rather rough as compared to the rigid technic employed in more exacting experiments (15, 16). This also applies to the total nitrogen value given for the daddy sculpin (Table II). From urinary chloride data upon flounder presented elsewhere (16), the high chloride and low total nitrogen values in the samples of flounder urine can be similarly interpreted. In line with this interpretation is the fact that specimen *Flounder 1*, containing urine from a batch of fifty flounders subjected to rather severe conditions and overcrowding in a flounder net, shows a very low total nitrogen and a very high chloride concentration. The total nitrogen content of the sample of goosfish urine (Table II) is well below the values reported by Grollman (18) for bladder urine from freshly caught specimens, is comparable with those given by Edwards and Condorelli (8), and is far above those reported by Marshall and Grafflin (35) and Smith (40), whose specimens were obviously quite diuretic.

The abnormalities in urine formation observed under experimental conditions are associated primarily with a derangement of the normal salt and water balance of the organism, resulting from the loss of water through the skin and the increased ingestion and gastro-intestinal absorption of sea water (42, 17). The important fact, for the present data, is that from all available evidence there is no concomitant derangement of nitrogen metabolism under these conditions; i.e., the decreased urinary concentration of total nitrogen represents primarily a dilution effect. This statement is borne out by a comparison of the nitrogen distribution data upon the urine of the goosfish previously reported by Grollman (18) and Smith (40), the former at high and the latter at low urinary total nitrogen concentration. Under the circumstances, the analytical data given in Table I are interpreted as representing fairly accurately the normal percentage distribution of the ordinary nitrogenous constituents and percentage of undetermined nitrogen in sculpin and flounder urine.

The ammonia and uric acid values for sculpin and flounder are quite low, and are comparable with those reported by Grollman (18) for *Lophius*, but much lower than those reported for ammonia in *Lophius* and *Muræna* by Edwards and Condorelli (8). Urea is present in considerable amounts in sculpin and flounder urine, some data on sculpin having been reported by Marshall and Grafflin (36). The

urinary excretion of urea in significant amounts seems to be characteristic of all marine teleosts so far studied except the goosefish (*Lophius piscatorius*). Marshall and Grafflin (35) reported that the plasma and urine of this species uniformly contain urea in only minimal amounts, and this was subsequently confirmed for the urine by Grollman (18) and Smith (40). The high values reported for *Lophius* by Edwards and Condorelli (8) seem to cast doubt upon all of their urea analyses. The amino-acid and total creatinine values for sculpin and flounder are to be compared with other data available in the literature (8, 18, 35, 37, 40). In the marine teleosts as a group, the total creatinine fraction of the urine consists predominantly of creatine. Fairly extensive data upon the creatine content of sculpin urine, under normal and diuretic conditions, have been supplied by Pitts (37).

It is evident from Table I that approximately one-half of the total nitrogen in the composite samples of sculpin and flounder urine is unaccounted for by the ordinary nitrogenous constituents. The

TABLE II

Trimethylamine oxide in urine of sculpin, flounder, daddy sculpin and goosefish.

	Sculpin 3	Flounder 3	Flounder 4	Daddy sculpin	Goose- fish
Total N, <i>mgm. per cent.</i>	98.5	66.2	65.8	44.5	212.0
(CH ₃) ₃ NO N, <i>mgm. per cent.</i>	22.8	-0.3	1.4	14.1	69.1
Total N, <i>per cent.</i>	23.1	—	—	31.7	32.6

demonstrated presence of trimethylamine oxide—(CH₃)₃NO—in the muscles of marine elasmobranchs and teleosts and particularly its presence in the urine of the goosefish (see Table III) made it appear likely that this compound might be present in the urine of our animals. Using the micro-method of Hoppe-Seyler (24), results were obtained which indicated the presence of the oxide in considerable amounts in sculpin urine, but its absence in readily detectable quantities from flounder urine. Since there was a rather long interval between the collection of these urine samples and their analysis for trimethylamine oxide, and since the oxide has been found to undergo a slow decomposition to trimethylamine even in well-preserved urine (18), these data were rejected and new urine samples were collected in the following summer. These samples were analyzed soon after collection (Table II) and confirmed the previous findings. The (CH₃)₃NO figure (22.8) for the fresh composite sample of sculpin urine (*Sculpin 3*)

is to be compared with the figure 20.4 (milligrams per cent of nitrogen as $(\text{CH}_3)_3\text{NO}$) obtained upon sample *Sculpin 2* (preserved with sulfuric acid) more than two months after collection. This latter value represents 24.1 per cent of the total nitrogen, 44.5 per cent of the undetermined nitrogen. Although present in sculpin urine in considerable amounts, trimethylamine oxide is apparently entirely absent from flounder urine, or present in only small traces. The amount present in the urine of the daddy sculpin is comparable with that in the sculpin; and the value for goosefish urine is essentially confirmatory of the results of Grollman (18).

In order to obtain additional evidence that the substance present in sculpin urine is actually trimethylamine oxide, this compound was isolated from urine sample *Sculpin 1* as the aurichloride. For trimethylamine oxide this salt would have the composition $(\text{CH}_3)_3\text{NO} \cdot \text{HAuCl}_4$. Synthetic material of this composition gave a melting point of $255-7^\circ$, which agreed exactly with that of the salt isolated from sculpin urine. The mixed melting point was likewise $255-7^\circ$. On analysis the sculpin salt gave the percentage of gold as 46.0, 46.25 (theoretical 47.5 per cent); percentage of nitrogen 3.5 (theoretical 3.4 per cent). From the aurichloride the picrate was prepared in the following manner. The gold salt was dissolved in water and treated with "molecular" silver until the yellow solution became colorless. The silver was then centrifuged off and slightly less than the calculated amount of picric acid solution was added. The melting point of the picrate from the sculpin gold salt and of synthetic picrate from trimethylamine oxide, as well as the mixed melting point, all agreed: $194-7^\circ$. In addition, the picrates from the natural and synthetic sources had the same crystalline form.

Thus there is adequate evidence that trimethylamine oxide makes up a considerable portion of the "undetermined" nitrogen fraction of sculpin urine. As to the nature of the compounds present in the remaining fraction of the undetermined nitrogen in this species, we have no satisfactory data. This applies also to the entire undetermined nitrogen fraction of flounder urine.

DISCUSSION

The presence of trimethylamine oxide and trimethylamine in the fishes has been the subject of considerable investigation, and the present state of our knowledge is summarized in Table III. Supplemental to the table is the clear demonstration of the presence of trimethylamine oxide in the blood of *Scyllium catulus* (24) and in the roe

of *Clupea harengus* (38).² It is obvious that our negative finding for the urine of the flounder, *Pseudopleuronectes americanus*, is particularly striking in view of the positive results obtained from the muscles or urine of all other marine teleosts so far examined, especially from the muscles of the closely related *Pleuronectes cynoglossus*. The generalization has frequently been made that trimethylamine oxide is characteristic of marine, as opposed to fresh water, teleosts. Its absence from the urine of the flounder does not, naturally, preclude the possibility of its occurrence in the muscles of this species.

TABLE III

The occurrence of trimethylamine oxide and trimethylamine in the muscles and urine of fishes.

	Muscles		Urine		References
	TrO	Tr	TrO	Tr	
Selachians (Marine)					
<i>Acanthias vulgaris</i>	+	+	+	-	21, 24, 43, 44
<i>Lamna cornubica</i>	+	+	-	-	21
<i>Scyllium catulus</i>	-	-	+	+	24
Teleosts					
a. Marine					
<i>Clupea harengus</i>	+	+	-	-	21, 38
<i>Gadus aeglefinus</i>	+	+	-	-	21, 25
<i>Gadus morrhua</i>	+	+	-	-	21, 25, 26
<i>Mullus barbatus</i>	+	+	-	-	21, 25
<i>Pleuronectes cynoglossus</i>	+	+	-	-	21, 25
<i>Pseudopleuronectes americanus</i>	-	-	0	-	This paper
<i>Conger vulgaris</i>	+	+	-	-	21, 25
<i>Lophius piscatorius</i>	-	-	+	+	18, this paper
<i>Myoxocephalus octodecimspinosus</i>	-	-	+	-	This paper
<i>Myoxocephalus scorpius</i>	-	-	+	-	This paper
b. Fresh water					
<i>Anguilla vulgaris</i>	0	0	-	-	21, 25
<i>Salmo salar</i>	0	0	-	-	21, 25
<i>Squalius cephalus</i>	0	0	-	-	21, 25
<i>Alburnus lucidus</i>	0	0	-	-	21, 25
<i>Perca fluviatilis</i>	0	0	-	-	21, 25
<i>Cyprinus carpio</i>	0	0	0	0	21, 24

+ = present; 0 = absent; - = untested; TrO = trimethylamine oxide;
Tr = trimethylamine.

² Grollman (18) concludes that the trimethylamine present in goosfish urine was probably absent from the fresh specimens, and resulted from the slow decomposition of the oxide; and Hoppe-Seyler (24) indicates the possibility of a similar origin for the amine present in his samples of selachian urine. He further states (23, 24) that one cannot for the present be certain that the small amounts of trimethylamine previously (21) found in extracts of fresh muscles of selachians and marine teleosts (and originally regarded as a metabolic rather than a decomposition product) do not arise from the oxide in the course of the working-up process. He could also detect no relationship between the presence of the amine and its oxide and seasonal variations or the spawning period.

Trimethylamine oxide has further been demonstrated in cephalopods (20) and, on the basis of Hoppe-Seyler's interpretation (22), in crustacea by Suzuki *et al.* (45, 46), who reported it as "kanirin."³ From the gonads of the jelly-fish, *Rhizostoma cuvieri*, Haurowitz (19) has isolated trimethylamine, which is accepted as existing preformed in the tissues (21). Neither the amine nor its oxide has ever been reported as occurring in the normal tissues or body fluids of amphibia, reptiles or birds. Kappeler-Adler and Krael (26) reported an indirect demonstration of the oxide in mammalian muscle, and Lintzel (32) has very recently claimed trimethylamine and trimethylamine oxide to be normal constituents of human urine.⁴

From a detailed consideration of the osmotic relationships existing between the fishes and their environment, Hoppe-Seyler (23, 24) concludes that the retention of nitrogenous metabolic products creates the conditions which are necessary for the formation of the oxide. Thus the selachians, which are able to hold the salt content of their body fluids much lower than that of sea water, establish isotonicity with their environment through the retention of urea, trimethylamine oxide and betaine, and thus hinder the withdrawal of water from the body by the surrounding medium. Actually, Hoppe-Seyler's conception of the existing relationships in marine elasmobranchs is erroneous. Selachian blood is hypertonic to sea water, and this group is to be classed along with fresh water fishes as osmotically superior to the environment (42). That there is an active conservation of the oxide by the kidneys has been established by his demonstration of the much lower concentration in the urine as compared with the blood. According to Hoppe-Seyler, in the marine teleosts a similar, but much smaller, retention of metabolic end-products must occur, and in this

³The closely related tetramethylammonium hydroxide has been isolated from *Actinia equina* (2).

⁴The presence of trimethylamine in human urine has often been reported (among others, 5, 10, 14); but following the investigations of Takeda (47), Kinoshita (27) and Erdmann (9) it has been generally accepted that the amine does not exist preformed in the urine, but is liberated from some precursor by either chemical or bacterial decomposition. Lintzel refuses to accept this conclusion, and goes on to show that when large quantities of trimethylamine oxide, betaine, choline, etc. are added to human urine, the yield of trimethylamine does not increase. He further reports that when urine is distilled for trimethylamine for several hours, a subsequent distillation yields no more of the substance, even though a considerable quantity of trimethylammonium base is present. Actually, Takeda concluded that preformed trimethylamine is perhaps present at times in normal human urine, though always absent from that of the dog and horse. Trimethylamine has likewise been reported in human cerebrospinal fluid (7) and in the blood of lower mammals (6, 7) and man (7, 14). However, these reports have all been subjected to the same criticism that the amine may well have arisen from some precursor through chemical or bacterial decomposition.

way the difference between the osmotic pressure of the sea water and of the blood is at least diminished. The validity of this argument has been questioned by Homer Smith, in an article to appear in *Biological Reviews*, on the ground that direct evidence for an active retention in marine teleosts has never been adduced. On the other hand, in fresh water teleosts, living in a markedly hypotonic medium, the conditions are such that metabolic products are withheld as little as possible, and the oxide never occurs (Table III).⁵ Hoppe-Seyler extends his argument to the presence of the oxide in cephalopods and of the betaines in marine invertebrates and in plants, and makes the inclusive generalization that wherever nitrogenous metabolic products are retained, the preliminary conditions for the occurrence of methylation processes are present.

The conclusion of this investigator (21, 23), that on the basis of all available data trimethylamine and trimethylamine oxide must be considered primarily as end-products of protein metabolism, is concurred with by Poller and Linneweh (38) and Grollman (18), and is confirmed by the demonstration of the oxide in the urine of selachians and marine teleosts (23). This is in agreement with the general view expressed by Kutscher and Ackermann (29, 30) for the methylated muscle bases. Hoppe-Seyler (21, 23) regards trimethylamine and its oxide as derived primarily from ammonia through methylation and oxidation; and sees in the formation of the oxide the replacement of ammonia, which is toxic for the vertebrates in high concentration, by an essentially non-toxic, very weakly basic compound. The other methylated muscle bases, on the other hand, most probably are derived from higher degradation products of proteins, the amino acids (30). The idea that trimethylamine and its oxide may arise from the betaines, cholines (lecithin) and other compounds has also been advanced (18, 21, 28), and Lintzel (32) accepts such an origin in the human through the activity of the intestinal bacteria (see below).

The possibility that trimethylamine and its oxide are, in the fishes, in part at least of exogenous origin, being ingested preformed, has been

⁵ Hoppe-Seyler (23) sought to settle the question whether trimethylamine and its oxide actually do appear as metabolic products in fresh water teleosts, but are not demonstrable because of their rapid and complete excretion in the dilute urine. Carp were allowed to swim about in limited quantities of water over a period of seven days, and the entire body of water (containing the urine), when concentrated to small volume and treated with zinc dust and HCl, gave no precipitate with gold chloride. The logical conclusion was that the amine and its oxide are not formed in fresh water fish. Smith (40) reported a volatile base, in addition to ammonia, in the branchial and urinary excretions of the carp, and concluded that it was presumably in large part trimethylamine liberated from the oxide on treatment with zinc dust and alkali. The inability of Hoppe-Seyler to detect either the oxide or the free amine in the urine or muscles of this species (21, 24) renders Smith's interpretation untenable.

accepted by Grollman (18) and Hoppe-Seyler (21). The latter, however, believes it impossible that this could form the only source for the amounts demonstrable in selachians and marine teleosts, even though one assumed a strong retention of nitrogenous metabolic products. Lintzel (32) similarly accepts an exogenous origin for part of the trimethylamine and trimethylamine oxide in human urine. According to the latter investigator, ingested trimethylamine would appear in the urine principally as the oxide.

Hoppe-Seyler (21) has concluded that one must consider the possibility that in the fishes trimethylamine and its oxide can, at least in part and under certain conditions, be demethylated. From the work of Löffler (33), who showed an increase in urea formation when the surviving liver was perfused with trimethylamine, it is reasonable to assume that demethylation is a normal process in the mammals.

Suwa (44) first showed that in the presence of putrefying tissue trimethylamine oxide was converted to the free amine; and Poller and Linneweh (38), on the basis of their demonstration of the oxide in the muscle of *Clupea harengus* and this known bacterial conversion, offered an explanation for the large amounts of trimethylamine known to be present in herring brine. Ackermann and Schütze (1) and Kohlrausch (28) demonstrated the liberation of the amine from lecithin, choline and betaine under bacterial influence; and Takeda (47) demonstrated the appearance of trimethylamine in considerable amounts in mammalian urine allowed to undergo ammoniacal fermentation. Later, Ackermann, Poller and Linneweh (3) showed that the conversion of trimethylamine oxide to the amine, demonstrated by Suwa with putrefying tissue, could also be effected by fresh liver, even when cooked, indicating in the latter case a non-fermentative reductive mechanism. Lintzel (32) discusses the assumption that a bacterial decomposition of some of the trimethylammonium bases occurs in higher organisms; and concludes, on the basis of extensive feeding experiments with betaine, choline, lecithin, γ -butyrobetaine, carnitine and meat extract, that trimethylamine and its oxide, insofar as they are not ingested preformed, must owe their presence in normal human urine to the activity of intestinal bacteria upon certain of these bases, liberating trimethylamine, which would then appear in the urine for the most part as the oxide (see below).

In feeding experiments on human beings, Lintzel obtained the surprising result that ingested trimethylamine appears almost quantitatively (within 10 per cent) as trimethylamine oxide in the urine, and concludes that we must designate the oxide as the normal end-product

of trimethylamine metabolism in man.^{6, 7} On feeding preformed trimethylamine oxide (both as the pure compound and as marine teleostean muscle), he found the majority of it excreted unchanged in the urine, with a moderate rise in trimethylamine.⁸ He commented upon the rough proportionality between the oxide and the free amine in the human as indicating some sort of physiological relationship, perhaps with respect to oxidative-reductive processes in the organism; and cited in support of this idea the *in vitro* experiments of Ackermann, Poller and Linneweh (3), who stressed the biological importance of the oxide as a hydrogen-acceptor, particularly with respect to the sulfhydryl group of reduced glutathione.

It is a pleasure to acknowledge the capable assistance of Gordon Spence, who accepted full responsibility for the laborious task of catching, handling and collection of urine from the sculpins and flounders used for Specimens 1 and 2.

SUMMARY

It has been shown that approximately half of the total nitrogen in the urine of the sculpin (*Myoxocephalus octodecimspinosus*) and the flounder (*Pseudopleuronectes americanus*) cannot be accounted for by the ordinary nitrogenous constituents. Trimethylamine oxide is present, and accounts for a large portion of this unknown fraction in the sculpin, but is either absent or present in only very small quantities in the urine of the flounder. Brief supplemental studies demonstrated the presence of trimethylamine oxide, in considerable amount, in the urine of the daddy sculpin (*M. scorpius*) and confirmed its presence in the urine of the goosefish (*Lophius piscatorius*). In the discussion the literature concerning the occurrence and significance of trimethylamine oxide and trimethylamine is reviewed.

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⁶ Interesting in this regard is the finding (24) that when trimethylamine was injected into carp (in which neither the amine nor its oxide normally occurs) it could be recovered in part from the urine, but that no trimethylamine oxide was formed.

⁷ Langley (31) had previously claimed that rabbits metabolized the large majority of ingested trimethylamine, the remainder being excreted unchanged in the urine. He further concluded that part, if not all, of the metabolized amine seemed to be excreted as urea.

⁸ Suwa (44) obtained essentially similar results on feeding experiments with rabbits. When the oxide was injected subcutaneously, on the other hand, it was excreted in part as trimethyl- and dimethylamine, but he could detect no unchanged oxide in the urine.

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